Original Research Article

Investigation of slow freezing of human testicular tissue on proliferation, colonization and viability of human spermatogonial stem cells

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ABSTRACT

In this study, the effects of freezing of testicular tissue by slow freezing on the proliferation rate, colonization and viability of spermatogonial stem cells (SSCs) were evaluated. The testicular tissue of the freezing group was freezed by slow-freezing method, and the fresh group remained intact. The results showed that the cell proliferation rates of fresh group [1.48 ± 0.02] and [1.77 ± 0.02] compared to the frozen-thawed group [1.39 ± 0.02] and [1.64 ± 1.64] in the 7th and 14th days, were significantly higher. There was significant difference between the number of colonies in the 7th and 14th days. In the first day after cell isolation, cell viability of the fresh group [95 %] was significantly higher than the frozen-thawed group [87.7 %]. Also, in the 7th and 14th day, cells viability fresh group were significantly more than frozen-thawed group. These results suggest that slow freezing of testicular tissue reduces the proliferation, colonization and survival of SSCs.

***Keywords*:** Slow freezing, testicular tissue, spermatogonial stem cells

INTRODUCTION

Long-term preservation of the spermatogonial stem cells [SSCs] for further use in research, understanding the biology of SSCs, and ultimately transplanting these cells to induce and resume the spermatogenesis process is very important [1]. Long-term preservation of these cells can be achieved by either maintaining SSCs or maintaining testicular tissue containing these cells [2]. SSCs are very important as the only cell capable of transferring genetic traits from generation to generation, and maintaining and reusing these cells, especially for those who have cancer and who are being treated with chemotherapy drugs, and or radiotherapy is very valuable and important [3]. The long-term preservation of SSCs has been successfully reported by various researchers in many species and in different ways [4,5]. Two common methods for freezing testicular tissue are slow freezing and verification methods. The method of slow freezing compared with verification has been used more in studies, although both methods are used to freeze testicular tissue [6]. Slow freezing is done using a machine that gradually reduces the temperature. The tissue parts are ultimately exposed to a cryoprotectant solution at 4 °C for 15 min and then placed in a controlled temperature to freeze. Then they were placed inside the cryovials and kept in liquid nitrogen [7]. There is another protocol for this method, which was done by Yildia in 2013 using a programmed device to place mice testicles. After the tissue components were placed in a 4 °C equilibrium solution, they cooled at a temperature of 1 °C/min to 0 °C and then maintained for 5 min. Then they cooled at -0.5 °C/min to 8 °C. After 15 min at this temperature, the temperature reduction was maintained at -0.5 °C/min until the temperature of 40 °C was maintained and this temperature was maintained for 10 minutes. The tissues were then cooled at -7 °C/min to 80 °C and immediately transferred to liquid nitrogen [8]. This method makes the tissue less exposed to damage from cryoprotectant. The successful freezing of testicular tissue depends on the choice of cryoprotectant, its density, and the type of freezing and melting method. In a study of slow freezing for the immature of immature human testicles, DMSO was reported as the best cryoprotectant between other cryoprotectants, including ethylene glycol, propanediol and glycols. This study, based on histological observation, resulted in the maintenance of the structure along with the survival of the existing cells, including spermatogonial, sertoli, and interstitial cells [9,10]. Jahnukainen froze the testes of the

monkey with DMSO in 2007, and after melting transplanted to the mouse body. In their results, reported the resumption of spermatogenesis after xenotransplantation [11]. These reports, together with previous results, determine the preferred DMSO as a suitable cryoprotectant for freezing the testicular tissue. Some previous studies, although not clearly communicating their beneficial effects, used DMSO with sucrose to freeze testicular tissue. However, the effect of slow freezing on the quality of cell culture of isolated SSCs from the frozen-thawed human testes is important in evaluating the use of these cells.

MATERIALS AND METHODS

Isolation, culture and confirmation of the identification of SSCs

Testicular biopsy from the Gandhi infertility center were obtained from patients with obstructive azoospermia, after the completion of the therapeutic phase, from the rest of their sample. All stages of this study were performed according to the approval of the research ethics committee of the Tarbiat Modares university faculty of medical sciences. Patient biopsy samples in DMEM medium (Gibco, UK) containing L-glutamine (Gibco, UK), 10 % FBS (Gibco, UK), sodium bicarbonate (Gibco, UK), nonessential amino acids, Penicillin and Streptomycin (Gibco, UK) were transferred to the laboratory for a maximum of one h. Samples were prepared after washing in PBS (Invitrogen, UK) for the separation of spermatogonial cells. For isolation of spermatogonial cells, the protocol which were subjected to two stages of enzyme digestion [12], were used with a few changes. Thus, the biopsies in a medium containing collagenase type 4 (Sigma, USA), hyaluronidase, trypsin [Sigma, USA], each of 0.5 mg/ml and DNAse 0.05 mg/ml completely cleaved and dissected with Pens and scissors and incubated for 30 to 45 min at 37 °C. During this time, the medium containing samples was pipetted several times by pipetting. In this phase, seminiferous tubules were isolated from surrounding connective tissues. Then, the tubes were allowed to deposit with a gravity gradient centrifuge (1500 rpm for 5 min). Then the top solution, which contains mostly blood cells and connective tissue, was drained, and in order to remove more blood cells, was added 3 ml of PBS to the plate, and again centrifuge and re-soluble solution with 1000 rpm for 3 min. Evacuated the result of the first phase of enzyme digestion at the end was the parts of seminiferous tubules that entered the second stage of digestion for further digestion. To the settled plaque, a fresh culture medium containing the above enzymes was added and pipetted for 10

minutes at 37 °C. In this phase, seminiferous tubules are disintegrating and all cells in the tubes, including sertoli cells, are released. This suspension was then washed out as the first stage of centrifuge with PBS. Ultimately, the top solution was evacuated and the plaque formed was diluted and ready to be cultured with a culture medium. The cell suspension was made up of spermatogonial cells with sertoli cells and was used for two weeks. In order to culture these cells, the amount of 5×10^5 cells per well of 6 wells containing αMEM (Bio-Ideal, IRI) with 10% FBS (Gibco, UK) plus penicillin-streptomycin antibiotics in an incubator it was placed at 32 to 34 ° C and 5 % CO_2 for two weeks. The cell culture medium was changed every 48 h.

Immunocytochemistry

All of cells were first cultured on sterile lamellas in a Petri dish. After 24 h of culture, the cells were fully primed to immunocytochemistry. The cells were also cultured for 6 days and used for immunocytochemistry after colonization of SSCs. The method of doing the work was based on the study of Mohaqiq [13]. First, the colonies formed on the lamella were washed twice by PBS for 5 min. For fixation, they were placed in 4 % paraformaldehyde (Gibco, UK) for 20 min. The washing followed by PBS for 5 min. For intra-cytoplasmic and intra-nuclear antigens, 5 mL of triton 100 X 2 % (MP Biomedical, USA) was used to facilitate the penetration of antibody into the cell. The inhibitory step in 10 % Goat serum (Vector Laboratories, USA) was performed for 30 min at 37 °C. The colonies were then added to the primary antibody and kept at 4 ° C for 24 h. After this step samples were washed three times by PBS for 5 min. Then secondary antibody tailored to the primary antibody in dark and room temperature were added for 2 h. After three times washing with PBS each time for 5 min, the samples were glued together with glycerol phosphate.

Freezing of testicular tissue with Planner

For the freezing and melting of the testes, the protocol of Zeng [14] were used. In this way, the small parts of the testicular tissue were placed in a freezing culture medium and then placed in a programmable freezer (Planner Cryo 360. 1.7-UK). The machine automatically processes all the freezing steps slowly and stepwise, and finally the frozen tissue is ready to leave the machine and put in liquid nitrogen.

How to work with the Planner

Testes biopsy were first scraped with scissors and then inside a 1-ml cryovials of Leibovitz-L-15 (Gibco-UK) containing 2 % FBS and 10 % DMSO (Sigma-USA). The tissues were incubated at room temperature for 15 min and then placed in a programmed freezer. The

testicular tissues were cooled at 20 °C to 4 °C at 2 °C/min. At this stage, seeding was done manually using a penny that was previously placed in liquid nitrogen. The temperature was cooled from 4 °C to 30 °C at a temperature of 0.3 °C/min and then cooled down from 30 °C to 130 °C at 10 °C/min. The vials were immersed in liquid nitrogen and then transferred to the storage chamber and held for 48 h. To melt, the vials are kept at room temperature for 1 min and then placed in a 25 °C bath for 1 min. One ml of Leibovitz L-15 medium was added to each vial and transferred to sterilized centrifuge tubes. To remove free radicals, the testes are washed twice with the Leibovitz L-15 medium.

Evaluation of the proliferation and colonization of SSCs

The number of spermatogonial cells was counted and compared in different days with the aid of a neubauer chamber. Evaluation of colonization was carried out based on previous studies [15]. Colonies derived from SSCs on the 7^{th} and 14^{th} day were evaluated for number and diameter with reverse microscope (Ziess, Germany) equipped with ocular grid. The viability of cells was measured on the first, 7^{th} and 14^{th} day by trypan blue.

RESULTS

Isolation and culture of fresh and frozen human SSCs

Testicular biopsy of patients with obstructive azoospermia were placed in two fresh and frozen-thawed groups and subjected to two stages of enzymatic digestion. In the first stage, the interstitial tissue was removed (figure. 1A), and in the second step, under the influence of enzymes, crushed tubes (figure. 1B) and suspended from germ cells and sertoli cells (figure 1-C) . The cell suspension from the enzymatic digestion caused numerous colonies in the cell culture system due to the presence of SSCs (figure. 1-F).

Confirmation of the identification of SSCs

The nature of colonies containing spermatogonial cells was shown using the immunocytochemistry method for *PLZF* and *Oct-4* proteins [16]. The results showed the expression of these proteins in these colonies (figure 2).

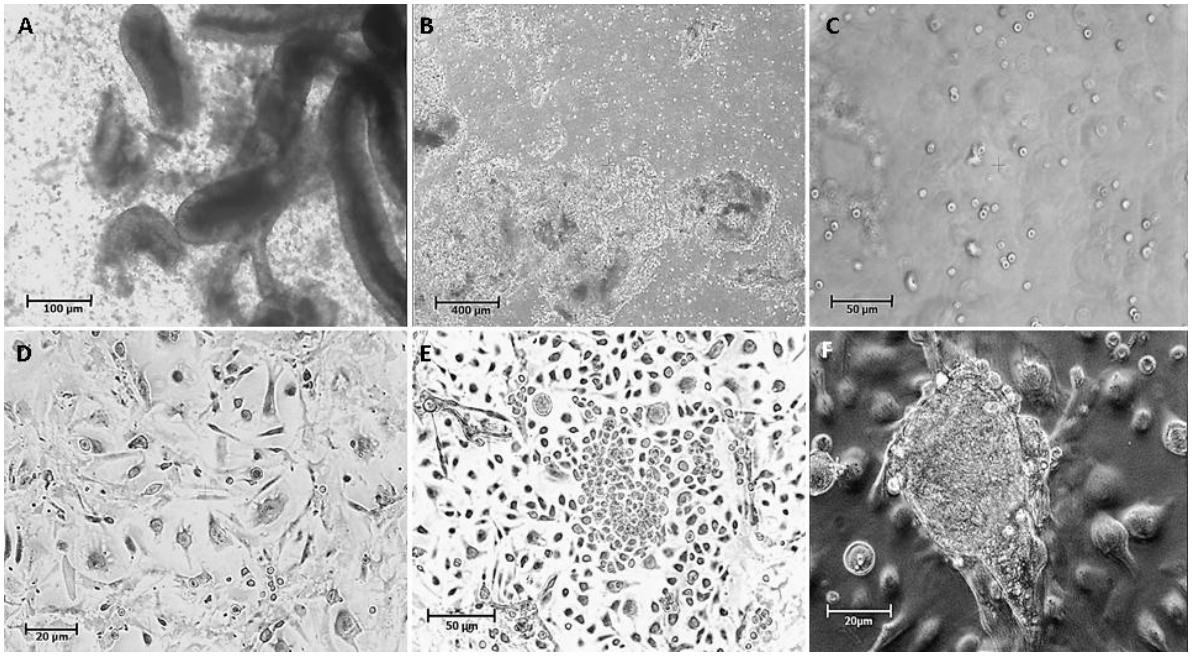

Figure 1. Human spermatogonial cell isolation process: interstitial tissue digestion [A], basal membrane digestion and residual interstitial tissues [B], cell suspension containing spermatogonial cell and sertoli cells obtained after two digestive step [C] progression cell division and cloning formation: spermatogonial and sertoli cells in co-culture conditions [D]. The spermatogonial cells make mitotic divisions close to each other [E]. Typical colony with a specific border [F].

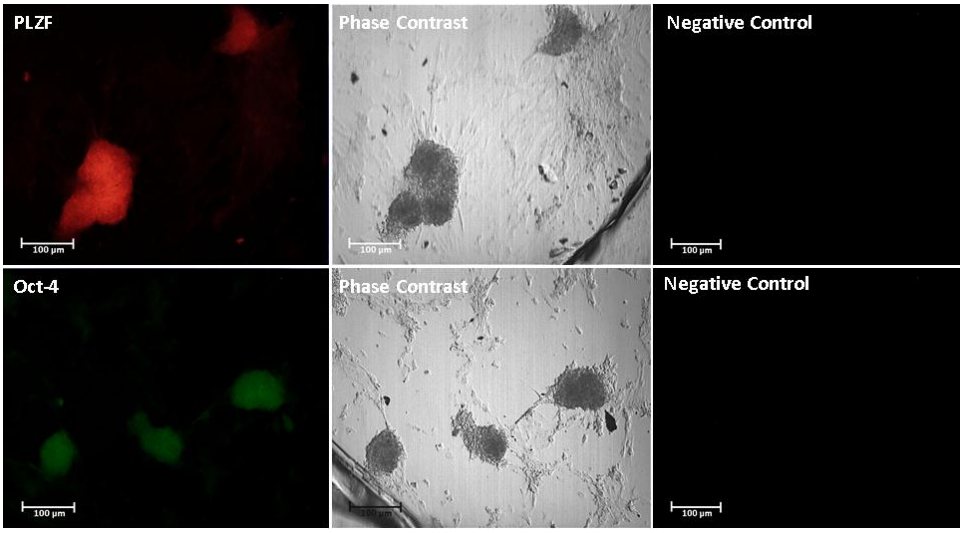

Figure 2. Confirmation of the nature of SSCs isolated from human testicular tissue. Expression of *PLZF* and *Oct-4* proteins in colonies derived from spermatogonial cells culture. Negative control is without primary antibodies.

Colonies derived from the culture of spermatogonial cells containing various classes of spermatogonial cells, which the SSCs present in these colonies, express the positive expression of these proteins.

Results of Proliferation, colonization and Viability of SSCs

On the 7^{th} and 14^{th} days, there was a significant difference between the mean of proliferation in different groups. Thus, on the 7^{th} and 14^{th} days, the proliferation rate of the fresh (1.48 ± 0.02) and (1.77 ± 0.02) in comparison with the frozen-thawed group cells (3.39 ± 0.02) and (1.64 ± 0.66) were significantly more (figure 1). The results of colonizing studies include comparing the number and diameter of the colonies obtained from spermatogonial cells on the 7^{th} and 14^{th} day in fresh and frozen-thawed groups, for 5×10^5 cells, the number of primary cells for cell culture (figures 2 and 3). On the 7^{th} and 14^{th} days, there was a significant difference between the mean number of colonies in different groups. Thus, on the 7^{th} and 14^{th} day, the number of colonies in the fresh group (4 ± 0.09) compared to the frozen-thawed group (1.3 ± 0.5) was significantly higher. On the 7^{th} and 14^{th} days, there was no significant difference between mean of colonies diameter in both groups. The results of the study on the viability of cells showed that there was a significant difference between the mean of cell viability in the first, 7^{th} and 14^{th} days in different groups. On the first day after the completion of cell isolation, the viability of the fresh group (95 % ± 0.9 %) was significantly higher than that of the frozen-thawed group (87.7 % ± 1.9 %). Also, on the 7^{th} and 14^{th} day, the rate of vitality of the fresh group (91.7 % ± 1.4 %) and (87.7 % 2.4 %) compared to the frozen-thawed group (84.0 % ± 3.2 %) and (82.7 % ± 3.2 %) were significantly higher (figure 4).

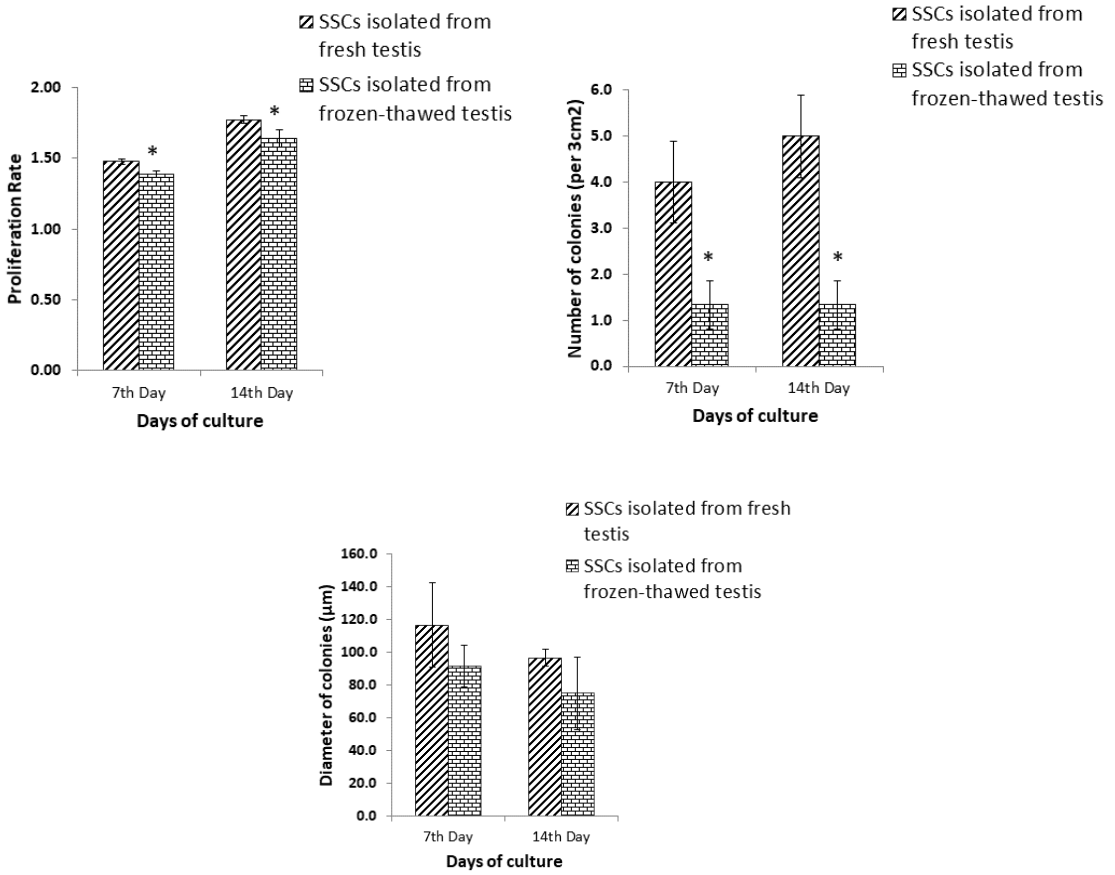

Figure 3. Comparison of proliferation rates on the 7th and 14th days of cell culture in different groups. Results are reported as mean ± standard deviation. Experiments were repeated at least 3 times for each group and comparison the diameter of colonies on the 7th and 14th day of cell culture in different groups Results are reported as mean ± standard deviation. Experiments were repeated at least 3 times for each group.

DISCUSSION

Freezing is a method which cells could be stored for a long time. In fact, one of the most important developments in the germ cell transplantation process is the induction of spermatogenesis with frozen-thawed germ cells, many studies have been done [17,18]. One of the most important use of this technique is clinically in patient with cancer and in young people who should undergo severe chemotherapy that often reduces the amount of sperm production in the long-term and sometimes irreversibly. In immature boys who have not yet started sperm production, their spermatogenesis activity can be maintained against radiotherapies by freezing SSCs in liquid nitrogen and reintroducing them into testicles. Izadyar in 2002, examined various methods of freezing

and cryoprotectants, providing an effective method for the freezing of SSCs. They showed that freezing in a culture medium containing 10 % FCS, 10 % DMSO and 0.07 M sucrose using an uncontrolled freezing method is the easiest and best way to maintain and maintain spermatogonia A, which after melting, 70 % of cells survived and propagated in culture medium. Also, these cells retained their function and were able to create colonies after transplantation in the rat testis [4]. In a recent study by Mirzapour in 2013, human SSCs derived from azoospermic patients were frozen and thawed using DMSO as cryoprotectant and a combination of fresh Sertoli cells. In this way, the number of colonies obtained was significantly higher than the time when the cells were cultured alone [5]. The testicular tissue consists of stem cells and various types of somatic cells, including sertoli cells, laydig cells, myoid, vessels, and interstitial connective tissues. Because different cell types have different freezing conditions and requirements, and in order to freeze each individual cell, they require a high permeability to a cryoprotectant agent. The testicular tissue freezing is challenging due to its complex structure. A team of researchers reported that spermatogenesis was resumed after transplantation of frozen-thawed testis tissue, and the sperm obtained by microinjection, embryo was formed and the offspring was born [19,20]. However, due to the relationship between the seminiferous tubules membrane with Laydig cells, lymphatic cells, and blood vessels that vary among different species, the freezing protocol for mouse testicular tissue may be for other tissue types Mammals are not suitable. Keros in 2005 and Wyns in 2007 reported that the human testicular tissue was freezed and thawed by slowly freezing and maintaining the structural characteristics of testicular tissue. However, none of the previous studies achieved the in vivo spermatogenesis potential of the tissue after thawing [10,19]. In the present study, we used Zeng method to find a survival rate of 87 %. Based on the same results, the slow-release freezing method of testicular tissue seems to be a suitable method for the long-term maintenance of SSCs.

CONCLUSION

The slow freezing protocol has an acceptable reduction on the proliferation, colonization and viability of the cells. However, this protocol seems to be appropriate for long-term maintenance of testicular tissue and SSCs, although the improvement in this protocol is felt.

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